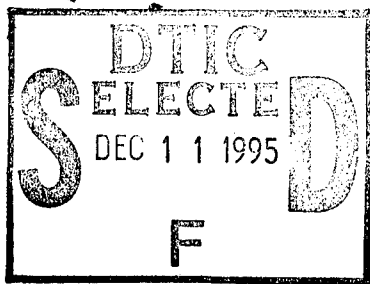


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#### IV. TABLE OF CONTENTS

I. FRONT COVER .....	1
II. SF298 REPORT .....	2
III. FOREWORD.....	3
IV. TABLE OF CONTENTS .....	4
V. INTRODUCTION .....	5
Background .....	5
Importance of the T cell response .....	5
T cell response to antigen.....	6
T cell subclasses and the measurement of cytokines .....	7
Methods for measuring CMI .....	7
Q Fever and the role of cellular immunity.....	8
Phase I Technical Objectives and Technical Approach .....	9
VI. MATERIALS AND METHODS .....	9
Luminometer .....	9
Luciferin-luciferase .....	10
Magnetic particles and separators. ....	10
Preparation of mononuclear cells. ....	10
Antibodies .....	11
Mitogens and antigens. ....	11
Immunization of mice.....	12
MTT and BrdU Assays.....	12
Flow Cytometry.....	12
VII. RESULTS .....	13
Development of a prototype (Specific Aim #1).....	13
Overview of the prototype assay format. ....	13
Bioluminescent measurement of ATP in lymphocytes.....	14
Immunomagnetic separation of cells .....	15
Alternative methods for preparation of cells. ....	17
Assay performance. ....	17
Measurement of CMI in Q Fever (Specific Aim #2).....	18
Comparison with standard assays for CMI (Specific Aim #3). ....	19
Use of the assay with human samples (Specific Aim #4). ....	20
VIII. CONCLUSIONS .....	23
Improvements and future work .....	24
Improvements in the basic format.....	24
Studies comparing this assay with other assays of CMI ....	26
Extensions and expansions of the method .....	26
Applications of the methodology .....	26
IX. REFERENCES .....	27

## V. INTRODUCTION

Cell mediated immunity (CMI) is a critical part of the immunological response to many infectious, neoplastic, and autoimmune diseases. A number of different assays have been used to measure various aspects of CMI such as lymphoproliferation and cytotoxicity *in vitro* and delayed type hypersensitivity skin testing *in vivo*. These assays are complex to perform, take several days to be completed, often give variable results from lab to lab, and are frequently difficult to interpret. Delayed type hypersensitivity is performed in a clinical setting, but this assay is at best semi-quantitative. A flow cytometric method has recently been used to study lymphocyte function, but this method is measures only one sample at a time and requires complicated equipment and analysis. Because it is more difficult to measure, the role of CMI in disease processes and in the response to vaccines and immunotherapy is much less understood than is the role of humoral immunity. This report describes the development of a prototype for a sensitive, rapid, and quantitative assay for measuring antigen specific CMI based on a combination of immunomagnetic separation and bioluminescent measurement of ATP levels. The prototype assay is simple, quantitative, and relatively rapid and requires little hands-on time. The prototype assay was evaluated using samples stimulated with various mitogens and antigens and was tested for its usefulness in studying response to immunization with Q fever antigens using a mouse system. This technology may be very valuable in development of vaccines and immunotherapies, in diagnosis of infectious disease and in research on the role of cellular immunity in disease processes such as autoimmunity and neoplasms.

### A. Background

#### 1. Importance of the T cell response

The immune response can be divided into humoral and cellular components. The humoral response to pathogens or antigens is mediated primarily through the production of antibodies by B cells directed against antigens on the foreign body. The antibodies can act in a number of ways to clear the foreign organism or proteins from the system. Humoral responses have been extensively studied and are relatively easy to measure with specific and sensitive immunoassays for antibodies in plasma.

The second component of the immune response, the cellular or T lymphocyte response is more difficult to measure and is generally less well understood. Nonetheless, the importance of cellular immunity in the defense against any diseases can be demonstrated by a number of approaches (Kabilan, et al., 1994; Schupperet *al.*, 1993; Sher, et al). In many diseases induction of an active antibody response or the acquisition of passively acquired antibody to the causative agent does not correlate well with protection against the disease. With this approach, it is often assumed that if antibodies do not prevent signs of disease, then other immune mechanisms (cell-mediated or mucosal) must be important for protective immunity. In some viral diseases, antibodies are nonneutralizing and coexist with virus in the serum. In other cases, neutralizing antibodies may be generated systemically and may be effective against viremia, but do not offer protection against localized virus challenge-exposure. In such diseases, cell-mediated, mucosal, and humoral immunity may all play important roles in protective immunity.

The correlation between antibody concentrations and disease protection may vary

depending on the model used to study specific immunity. For example, antibodies to rotavirus are detected inconsistently in the serum, making it difficult to assess the correlation between humoral immunity and disease protection, but suggesting that immune mechanisms other than antibody production may be important. Protective immunity to *Pseudomonas aeruginosa* can be induced in mice that do not have evidence of a concurrent antibody response. Antibodies in the serum appear to play a role in providing protection against *P. aeruginosa*, but are not essential for protective immunity.

The importance of CMI in providing protection against specific diseases may also be assessed by suppressing a specific arm of the immune system and evaluating the severity of disease after subsequent challenge exposure. Pharmacologic suppression of CMI responses may be achieved by treating animals with drugs such as Cyclosporin A. The increased susceptibility to a wide array of infections that characterizes AIDS is a profound testament to the importance of the T lymphocytes in protection from infectious disease.

In addition to their role in protecting the organism from infectious agents, T lymphocyte response can also be partly responsible for disease in that a number of autoimmune diseases, septic shock, and transplant rejection are mediated in some cases by over response of the cellular component of the immune response. The sequelae of Lyme disease have been attributed to activation of self-reactive lymphocytes (Groeneveld *et al.*, 1994; Pollack *et al.*, 1991).

Vaccines have been developed for a number of diseases and are designed to confer protective immunity on a subject that has not been exposed to the pathogen. Vaccines are also being used increasingly as therapeutic agents, to boost or alter the immune response in a patient that is showing an inefficient response to a vaccine. Vaccines have typically been developed and monitored by testing the plasma for the presence of antibody that neutralizes the infectious organism. This has not been an entirely effective approach and in some cases such as with vaccines developed for RSV, the vaccine has actually increased the incidence of serious complications despite the induction of antibody by the vaccine (Bright, *et al.*, 1995).

Many studies have now shown that the effectiveness of a vaccine is in many cases related to its ability to induce cellular immune responses. (Ada, 1994) The effectiveness of influenza virus, hepatitis B virus, and brucella viruses may be dependent on their ability to induce cellular immunity (Cabral *et al.*, 1978;). The induction of cellular immunity may require a different form of vaccine or a different adjuvant or immunization route than does the induction of purely humoral immunity (Ada, 1994; Allison, 1995; Germain, 1994).

## 2. T cell response to antigen

Measuring the cellular immune response to an antigen is complicated by several factors including the fact that antigen is recognized by T lymphocytes in the context of histocompatibility antigens. Like the B lymphocyte system in which exposure to antigen results in expansion of a specific group of cells that manufacture immunoglobulin specific to the invader, the T lymphocyte recognizes and responds to specific antigen. T lymphocytes express a T cell receptor (TCR) through which it recognizes foreign antigen. When the TCR on a lymphocyte is engaged, the lymphocyte moves from a resting or quiescent state into an activated state. T cell activation proceeds through many cellular events including calcium mobilization, changes in phosphorylation, transcription of new mRNAs ultimately to expression of function and proliferation or expansion of



the clone.

### 3. T cell subclasses and the measurement of cytokines

Measurement of the cellular immune response is also complicated by the existence of multiple different classes of responding cells and different consequences from activation of the different effector mechanisms. The major classes of effector lymphocytes are distinguished by specific cell surface markers: CD4<sup>+</sup> helper T cells, CD8<sup>+</sup> cytotoxic T lymphocytes, and NK cells which exhibit some of the characteristics and markers of T lymphocytes but which do not exhibit the T cell antigen receptor.

T helper cells can also be classified into three distinctive subsets based on the cytokines they secrete (Mosmann & Coffman, 1989; Ferrick et al, 1994). TH0 cells are considered to be the precursors, and they mainly secrete interleukin-2 (IL-2). Following antigen stimulation, TH0 cells differentiate down two pathways such that cells with one of two possible cytokine profiles emerge. TH1 cells secrete IL-2, gamma interferon (IFN $\gamma$ ), and tumor necrosis factor (TNF $\beta$ ); and TH2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Minty *et al.*, 1993). Considerable cross-regulation occurs between these two subsets, IFN $\gamma$ , which is a product of TH1 cells, inhibits TH2 cell proliferation, IL-4 and IL-9 gene expression, and IL-4 function. On the other hand, IL-4 and IL-10, which are products of TH2 cells, can down-regulate TH1 cell responses. This interplay can result in a predominance of TH1 or TH2 cells following preferential stimulation of a particular TH subset (Parronchi *et al.*, 1992). TH1 responses are characterized by strong cell-mediated immunity and an IgG2a antibody response in mice. In contrast TH2 responses are usually associated with high-titer IgG1 and IgE antibody responses, but poor DTH reactions. TH1 cells in humans were recently shown to possess cytolytic activity (Salgame, et al., 1991).

Stimulation of a particular TH subset can be critical in determining the outcome of infection. Certain parasitic diseases, notably *Leishmania major* infection of mice can induce TH1 or TH2 responses depending on the genetic make-up of the infected mice, but only TH1 induction is associated with resistance (Sher et al, 1992). TH2 responses exacerbate the disease. In other cases, TH2 responses to some infectious agents, such as schistosomiasis, in mice and humans are critical for protection. Measurement and modulation of the various arms of the cellular immune response may be critical to the development of effective vaccines and immunotherapeutic modulations in autoimmune and neoplastic disease.

### 4. Methods for measuring CMI

Measurement of CMI has been accomplished by many different methods both *in vitro* and *in vivo* (Clough & Roth, 1995). Some methods are general in nature. That is, in their unmodified forms, they may detect a cellular response to microbial exposure, but may not offer information as to the specific cell populations involved. Examples of general methods include lymphoproliferation *in vitro* and delayed type hypersensitivity (skin tests) *in vivo*. Other methods measure a specific effector function of CMI such as cytotoxicity or release of specific cytokines (Elson, 1995). In addition, assays like flow cytometry may provide indications about the size of populations with certain cell phenotypes without providing a lot of specific information about the reactivity of the identified cells.

Lymphoproliferative assays are lengthy procedures that require multiple steps to complete. Typically antigen is exposed to appropriate antigen-presenting cells which are then fixed or irradiated. T lymphocytes or appropriate subsets are separated from peripheral blood, adjusted to a fixed cell density, and exposed to processed antigen on the presenting cells. After several days of expansion, the cells are exposed to a pulse of  $^3\text{H}$ -thymidine. Cells are harvested and the radioactivity measured. Typically, cells are also exposed to a T cell mitogen and the antigen specific response is measured relative to the mitogenic response. Various reagents, timing, and procedure can lead to significant variability in the result between labs.

The major drawbacks of the standard proliferative assay are that it requires extensive cell manipulation, it is long, and it requires radioactive isotopes. Preparation of antigen presenting cells is required as well as the separation of the T lymphocyte responding cells and of additional subsets to obtain a full picture of the response. The assay requires from 3 - 7 days to allow expansion of the limited number of responding cells. This has limited the usefulness of these assays in diagnostic situations and in monitoring vaccine trials (Lerous-Roels, et al. 1994).

Measurement of the earlier events in lymphocyte activation could be a useful approach to monitoring the T cell response. Alternatives to the standard assay have included measurement of calcium influx, measurement of the appearance of cell surface activation markers by flow cytometry and measurement of cytokine secretion (Elson et al, 1995). These assays all have some drawbacks as screening or rapid assays due to requirement for expensive equipment or in time and manipulations.

#### 5. Q Fever and the role of cellular immunity.

Q fever is a zoonosis caused by the rickettsia *Coxiella burnetti*. *C. burnetti* is a strict intracellular pathogen belonging to the family Rickettsiae. It is found in many parts of the world and usually associated with exposure to domestic animals. Recent cases in urban areas have been traced to transmission through cats. Q fever can be asymptomatic or it can lead to an acute flu-like illness whose symptoms include fever, granulomatous hepatitis, pneumonia, and meningoencephalitis. It can also become chronic with endocarditis as the main symptom and can result in death. Vaccines are being developed and have shown encouraging success in the prevention of disease in high-risk individuals (Reimer, 1993; Ackland *et al.*, 1994; Izzo, 1993).

*C. burnetti* has a unique antigen phase variation. The virulent phase I is isolated from natural or laboratory infections of animals and humans, whereas the avirulent phase occurs during serial passage of *C. burnetti* in cell culture systems. The phase variation appears to correlate with smooth and rough lipopolysaccharide changes. Individuals that are exposed generally show an antibody response with high levels of specific phase II antibodies appearing early in the acute phase of the disease and high levels of phase II antibodies in the chronic phase of the disease. The antibodies take approximately 7-15 days to appear and a definite diagnosis can be confirmed only by seroconversion or four-fold increase in the titer (DuPont, 1994).

Cellular immunity is thought to be important in the response to Q fever and may be relevant in determining the difference between acute versus chronic cases. The most significant implication of cellular immunity in the disease comes from studies in central Africa that have shown an increase number of symptomatic cases of Q fever in HIV positive individuals (Belec et al. 1993).

Cellular immunity has been shown to be an important component of the immune response and disease outcome in other rickettsial diseases with strong cellular immune responses following infection. The efficacy of a vaccine against Rocky Mountain spotted fever, *Rickettsia* was related in part to the cellular immune response (Dumler et al, 1992; Holland, et al. 1993).

## **B. Phase I Technical Objectives and Technical Approach**

The primary objective of the research proposed in Phase I research was to develop a prototype assay for measurement of CMI that was rapid, simple, quantitative, and sensitive. The approach to this objective was based on the principle that cells activated by exposure to specific antigen show an increased metabolic rate. Normal lymphocytes present in peripheral blood are in a resting state. When a resting lymphocyte is stimulated either with a mitogen or through interaction of its the T cell receptor with antigen expressed on an antigen presenting cell, the lymphocyte is activated. In most cases, this activation is associated with progress through the cell cycle. The increase in metabolic rate associated with activation can be measured rapidly and with high sensitivity using the bioluminescent reaction of luciferin and luciferase. Because all cells, not only lymphocytes or activated lymphocytes contain ATP, it is necessary to separate the cell population of interest before measuring the ATP levels. This can be accomplished by immunomagnetic separation. The basic concept of the prototype assay is shown in Figure 1 of the results section. Specific aims of the project include:

1. To determine the optimal conditions for measuring increased ATP in lymphocyte subsets including the kinetics of the reactions, the optimal concentrations of all components and the optimal buffer systems.
2. To develop a prototype system for measuring cellular immunity to Q fever including the appropriate concentration and type of stimulating antigen.
3. To compare the prototype assay with classical techniques for assessing cellular immunity including lymphocyte proliferation and cytotoxicity.
4. To validate the assay using samples from individuals who have been exposed to Q fever as well as normal unexposed samples.

## **VI. MATERIALS AND METHODS**

### 1. Luminometer

A Lumat LB 9501/16 (EG&G Berthold) luminometer was used in most of the experiments described in this report. It is based on a photomultiplier device that can quantitate single photons. The instrument is designed so that once a tube is added to the instrument, a background reading is taken and provided this reading is within range, 100 ul of a solution is automatically injected and photon counting is begun. Because the operations of this instrument are hard-wired, it was not possible to perform direct comparisons of some of the signal parameters.

For some of the initial experiments, a Lumac Biocounter 200 was used. In this system injection and initiation of signal detection are manual and the volumes of the materials and the ratios could be modified.

## 2. Luciferin-luciferase

Several different luciferin-luciferase(LL) preparations were used in the course of these experiments. Initial experiments used a purified preparation of luciferase combined with luciferin and containing  $Mg^{2+}$  and EDTA. (Sigma, MI, Catalog No. L 0633). This reagent is supplied in a vial containing 20 mgs of the mixture at optimal concentration ratios for measurement of ATP. The lyophilized mixture was reconstituted in sterile distilled water prior to use.

An LL mixture was also obtained from Analytical Luminescence, CA that was prepared in essentially the same condition. A lyophilized mixture of luciferin-luciferase at optimal concentrations with a total amount of 20 mg was provided. The mixture was reconstituted with 0.01 M HEPES buffer, pH 7.8. This preparation behaved identically with the preparation from Sigma discussed above.

A third preparation was also used. This preparation, obtained from Sigma, Catalog No. FL-AAM was received as a lyophilized solution containing optimal luciferase, luciferin, EDTA,  $MgSO_4$ , BSA and DTT in a tricine buffer. The manufacturer recommends that the solution be reconstituted in 5 ml of sterile distilled water. Concentrations of ATP as low as  $10^{-16}$  moles can be detected using this solution. In the prototype assay, this concentration of reagent was generally more than sufficient for the detection of ATP from the number of cells typically obtained in separations. To conserve reagent, this mixture was typically diluted 1:10 before use. The performance of this reagent was superior to that of the other reagents which may be attributed in part to the use of DTT and BSA as stabilizers. These reagents have been shown to increase the total light emission for a given concentration of luciferase and luciferin and to increase the half-life of the signal. In the absence of these reagents, light emission from LL peaks in about 1 second, and decays rapidly with a typical half-life of one minute. Inclusion of DTT and BSA in the reaction mixture increases the half-life to over 10 minutes without a decrease in the maximum intensity.

## 3. Magnetic particles and separators.

For magnetic separations, Bio-Mag beads (Advanced Magnetix, Mass) were used. In most cases, BioMag goat anti-mouse beads were used. These are magnetic particles approximately 1 micron in size covalently attached to goat polyclonal antibody directed against mouse IgG. The beads are supplied as a suspension of superparamagnetic iron oxide particles that respond well to weak magnetic fields but have no magnetic memory. Particles are supplied as 1 mg/ml solutions containing approximately  $5 \times 10^8$  particles/ml. In some cases, magnetic beads covalently attached to goat antibody directed against rat IgG or mouse monoclonal anti-CD4 antibody were used.

Magnetic separation was performed using a BioMag Separator Tube Rack (Advanced Magnetix, Inc.). This unit is a two piece set consisting of a plastic test tube rack that can accommodate 60 tubes of 12x75 mm with a bottom portion containing permanent magnets in a configuration so that the magnets are centered at the bottom of the tubes when the two pieces of the set are assembled.

## 4. Preparation of mononuclear cells.

Human mononuclear cells for these experiments were isolated either from source leuko-

cytes obtained from Gulf Coast Regional Blood Center or whole blood samples from paid volunteers collected in heparin from RH Typing Laboratory. Mouse mononuclear cells were obtained from whole blood collected by orbital bleeds collected through capillary tubes containing a heparin solution. Except where noted otherwise, peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Inc.) using standard methods (Current Protocols in Immunology, Wiley). Briefly, samples were diluted 1:1 with sterile RPMI solution at room temperature and placed in 50 ml conical centrifuge tubes. Three ml of Ficoll-Hypaque solution per 10 mls of sample was layered underneath the sample. The tubes were centrifuged for 20 minutes at 2000 rpm (900 x g) at room temperature in a Beckman tabletop centrifuge. The mononuclear cell layer is removed from the interface of the upper plasma layer and the lower Ficoll-Hypaque layer, placed in a second tube, diluted with RPMI-1640 containing 10% FCS and 10 mM glutamine and centrifuged at 1000 rpm to pellet the white cells. The cell pellet is resuspended in complete RPMI media and counted using a hemocytometer.

### 5. Antibodies

Mouse monoclonal antibodies directed against mouse or human CD4 and human CD3 were obtained from Devaron, Inc., New Jersey. Neither of these antibodies bind to the site that is bound by the human immunodeficiency virus. The monoclonal antibody directed against CD4 was used for flow cytometry in conjunction with fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG (Kirkegaard and Perry Laboratories, MD) but did not give good results for reasons that were not further investigated. Data from flow cytometry studies presented below used dual labeled antibodies with antibody to CD4 labeled with FITC and antibody to CD8 labeled with R-phycoerythrin (R-PE) obtained from Dako, Corp, CA. FITC labeled goat anti-mouse IgG was used as an isotypic control. In some cases, triple labeled antibodies (Becton Dickinson Immunocytometry Division) were used in which the third antibody (CD3) is labeled with Cy5-R-PE. Antibody to CD69 was obtained from Immunotech, ME. Unlabeled antibodies were prepared at a concentration of 0.2 mg/ml in sterile PBS.

### 6. Mitogens and antigens.

Natural, human interleukin-2 was prepared by Biotechnology Transfer, Inc. and is assayed by bioassay to contain 1000 IU/ml. In general, IL-2 was used at 10% or 100 U/ml. Phytohemagglutinin (PHA, M form) was obtained from Life Technologies, Inc, MD as a crude sodium chloride extract of *Phaseolus vulgaris*. This solution was provided in a custom, liquid format and used at 1 % final concentration. Tetanus toxoid (*Clostridium tetani*, prepared by formaldehyde inactivation of tetanus toxin) was obtained from CalBiochem as a lyophilized powder and was resuspended in sterile RPMI 1640 media.

Q fever antigen was obtained from Integrated Diagnostics, Inc., MD. Both Phase I and Phase II antigen were prepared from *C. burnetti* (ATCC VR615) Nine Mile strain. Phase II antigens were prepared in mouse L929 cells seeded with frozen antigen. Phase I antigens were obtained after passage in mice. Disrupted spleen cells from infected mice were incubated with L929 cells to produce Phase I antigens. Supernatants from infected cultures were harvested, organisms pelleted by centrifugation and resuspended in phosphate buffered saline with 0.1% formaldehyde. This material was tested by Integrated Diagnostics for activity by re-incubation with L929 cells and no infectious material was detected. This antigen was tested for the ability to

bind antibody to *C. burnetti* from patients in a dot assay format designed by Integrated Diagnostics, Inc. Phase I antigen was supplied at 1 mg/ml and Phase II antigen was supplied at 2 mg/ml. Even though the antigen was received at BTI in an inactivated form, the material was treated as potentially infectious in accordance with the appropriate NIH, CDC, and DoA guidelines.

#### 7. Immunization of mice.

Balb/c mice were obtained and housed by Biocon in accordance with all applicable standards for the care and treatment of animals. After a 10 day quarantine period, orbital bleeds were collected through heparin containing capillary tubes. PBMC were isolated from these bleeds using ficoll-hypaque density gradient centrifugation as discussed above. These cells were used to optimize the assay for use with mouse blood. In addition, the samples were tested for reactivity with Q fever antigen using a mixture of Phase I and II antigens.

Mice were then immunized with a 1:1 mixture of 100 ug of antigen and Freund's complete antigen. Test bleeds were obtained at 7 days, re-immunized at eight days, and test bled at 21 days after which the animals were sacrificed. While the initial bleeds contained adequate anti-coagulant to prevent clotting, the last bleeds were clotted when they were received and it was difficult to obtain cells. Samples from groups of 5 animals were combined for density gradient purification in the initial bleeds to obtain enough cells for optimizing the assay conditions and for testing multiple samples and doses. In later bleeds and in the spleens, no viable cells were obtained after density gradient centrifugation. It is not yet clear why this was the case.

#### 8. MTT and BrdU Assays

PBMC were plated in microwells (100 ul/well) at a concentration of  $1 \times 10^6$  cells/ml. Six wells were used for each condition. Tissue culture media, 1 % PHA solution, 100 U IL-2, or anti-CD3 antibody were added - 100 ul per well. After incubation for 24-96 hours, 10 ul of MTT solution at a final concentration of 0.5 mg/ml was added to each well. The plates were incubated for an additional 4 hours at 37°C, then 100 ul of solubilization solution consisting of 10% SDS in 0.01 M HCl. After overnight incubation at 37°C, the plate was read using a Dynatech microplate reading using a wavelength of 570 nm with a reference wavelength of 650 nm.

In one experiment, cell concentrations from  $10^7$  cells/ml to  $10^5$  cells/ml were used to determine the ability of the assay to detect a given number of cells.

Attempts to measure cell proliferation in the lymphocytes based on a kit for measuring incorporation of 5-bromo-2-deoxyuridine (BrdU) obtained from Boehringer Mannheim were not successful. The kit was used according to the instructions but no measureable signals were obtained. It is possible that either the fixation of suspension cells was inadequate or that too few cells were present used in these experiments to obtain signals.

#### 9. Flow Cytometry

Flow cytometry data was obtained using a EPICS Profile Analyzer from Coulter through Clinical Cytometry Consultants, Gaithersburg, MD. Several preliminary runs were performed using a two step staining procedure with the CD4 antibody used in the magnetic separations. Because these results were somewhat difficult to interpret, samples were prepared and stained with either a double color antibody for CD4/CD8 or triple color antibody preparation described above. In addition, cells were stained with CD69 antibody and with FITC-goat antibody to mouse

IgG. A two parameter histogram of log 90o side scatter (LSS) on the X axis vs forward angle light scatter (FS) on the Y axis was generated and a bitmap was placed around cells with the general characteristics expected for lymphocytes. This bit map was check against green and red fluorescent staining patterns. PBMCs were either unstimulated or stimulated with a mitogen for 24 hours prior to performing the analysis.

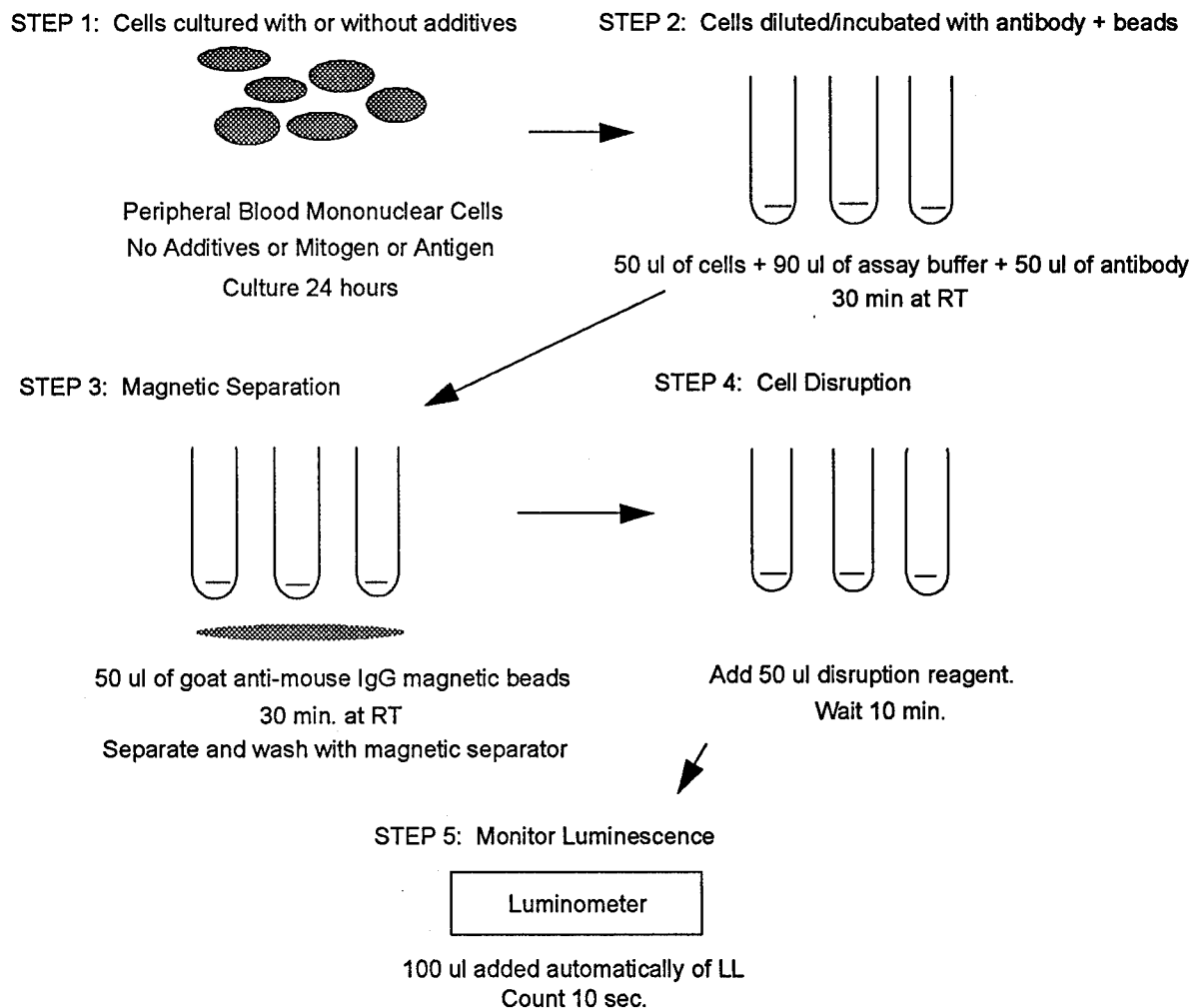
## VII. RESULTS

### A. Development of a prototype (Specific Aim #1).

#### 1. Overview of the prototype assay format.

A prototype assay was developed for the detection of cellular immune response to specific antigen. Figure 1 below shows the steps of the current version of the prototype assay.

FIGURE 1: PROTOTYPE ATP ASSAY FORMAT



Peripheral blood mononuclear cells isolated by gradient centrifugation over Ficoll-Hypaque are cultured either unstimulated or stimulated with specific antigen or mitogen for 24 hours. An aliquot of cells (50 ul) is added to 900 ul of wash buffer consisting of RPMI 1640 containing 10% FBS along with antibody to CD4 at a concentration of 2 ug/ml. Cells are incubated at room temperature for 30 minutes, then 100 ul of paramagnetic beads ( $1 \times 10^8$  beads/ml) are added. The cells suspension is gently mixed and incubated for 30 minutes at room temperature. The cells and beads are resuspended then placed next to a permanent magnet. After the beads form a dense pellet (approximately 3 minutes) the supernatant is decanted. The cells and beads are resuspended in RPMI with 10% FCS as a wash solution, then the magnetic separation is repeated. After fully decanting the media, cells in the bead pellet are lysed using a commercially available somatic cell lysis reagent (Sigma) and the tube is placed into the luminometer. The luminometer injects 100 ul of a luciferin:luciferase(LL) mixture.

Each cell treatment condition is run in triplicate as well as negative controls consisting of lysis buffer only or cells that are incubated with nonspecific beads, i.e., beads coated with a different isotype from that of the primary antibody. Finally, an ATP standard is run in each assay to confirm that the luciferin:luciferase reaction is occurring at appropriate levels. In some cases, controls were run both at the beginning and at the end of the experiment.

## 2. Bioluminescent measurement of ATP in lymphocytes.

The first experiments were designed to define the parameters related to measurement of total ATP concentration in peripheral blood mononuclear cells. In preliminary experiments, the effects of cell concentration, LL concentration, and lysis buffer composition on the light results were measured. As the last step in the assay, it was important to understand the parameters related to measurement of ATP in lymphocytes. For these initial experiments, mononuclear cells were prepared from mouse spleen as described above. The cell suspension was adjusted to a concentration of  $1 \times 10^6$  cells/ml in phosphate buffered saline (PBS) then 10-fold serial dilutions were prepared. In the initial experiments, equal volumes of cells and distilled water were mixed and 100 ul of luciferin:luciferase (Sigma -A solution, described above - diluted as described) was added and the relative light units were determined. Figure 2 shows the log of the relative light units as a function of the total number of cells. As can be seen, the log of the light output is directly proportional to the number of cells present over a broad range of cell concentrations. As few as 200 cells could be reliably distinguished from the background.

There is a complex relationship between the cell concentration and the LL concentration. As the LL concentration is increased above about 20 mg/ml, the background increased without a great increase in the signal. The optimal concentrations of LL solution were roughly 20 mg/ml although reduced levels gave overall reduced signal. In most of the experiments outlined here, LL was used at 2 mg/ml to conserve reagent. Additional increase in signal might be anticipated at higher levels of LL for the same number of cells.

Initially cell pellets or total cells were disrupted by adding distilled water. While this is adequate for some applications, variations in the total volume fluid containing the cell suspension and the composition of this fluid means that the total amount of cell lysis and the speed of the lysis vary from sample to sample. To overcome this issue, a cell lysis solution was used. This lysis solution contained a detergent in a buffering solution compatible with LL.

The magnetic beads had no effect on the signal, as the beads could be left in the tube with no



impact on the total signal either in the negative control (lysis buffer alone) or in the positive control (ATP solutions).

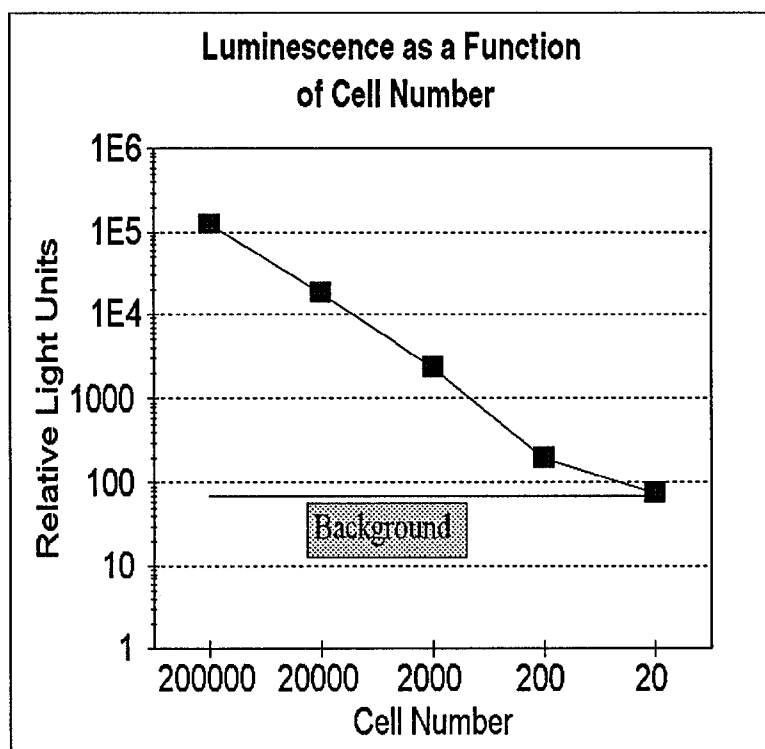


Figure 2: Sensitivity of Bioluminescence for Detection of ATP in PBMC.

Mononuclear cells isolated from normal mouse spleens were separated by density gradient centrifugation and counted. Dilutions of cells were prepared, lysed with distilled water, and 100  $\mu$ l of a 20 mg/ml LL solution was added. Relative light units are based on 20 sec counts using Lumac Biocounter 200. Background is signal of same LL solution with 100  $\mu$ l of media containing no cells.

### 3. Immunomagnetic separation of cells

#### *a. General procedure*

The total signal from the whole population could be measured by adding lysis buffer directly to an aliquot of the cells without immunomagnetic separation. In most cases, the ATP signal of the separated cells was a fraction of the total signal (roughly 10%) and the total signal for stimulated and unstimulated cells was roughly equivalent for at least the first 24-72 hours even though there were significant differences between the ATP signal from unstimulated and stimulated cells following separation. This is due, at least in part, to the fact that only a fraction of the peripheral blood mononuclear cells (PBMCs) are CD4<sup>+</sup> lymphocytes. The fraction of CD4<sup>+</sup> lymphocytes that are responsive to antigen have been estimated to be only a small fraction of the total population.

The beads and the primary antibody were used in saturating conditions for the total number of cells used. Increasing the concentration of the beads or the primary antibody did not increase the total signal following immunoseparation. Cells were generally cultured at a density of approximately  $5 \times 10^5$  cells/ml. Significantly lower and higher cell concentrations could be used and relative signal:background ratios were similar for mitogen stimulated cells.

While significant for the performance, the magnetic separation step was also one of the major sources of variability. When aliquots from a large pool of cells were separated, the initial results were quite variable suggesting that a large amount of the assay variability occurs in the separation step.

This could be due to several different factors: nonspecific trapping of cells by the beads, loss of variable numbers of beads during washing, and nonspecific binding of ATP or cells to the beads.

In theory, the magnetic separation of cells should take place in a direction perpendicular to gravity so that the intrinsic settling of the beads does not trap additional cells. Commercial magnetic separators are unsatisfactory for this type of assay because they require tubes that do not fit into the Luminometer or because the number of samples that can be separated simultaneously is very small. The magnetic separator utilized 96 10x 75 tubes but the magnetic source was placed at the bottom of the racks and consequently the tubes. In this configuration, cells trapped nonspecifically by gravity contributed to the background.

The mechanics of washing the beads was also problematic. Suction, no matter how gentle, resulted in loss of a significant and extremely variable proportion of the beads. This was solved by decanting the wash fluid from the beads, but this type of procedure is not ideal for a clinical lab or for use with many samples.

Finally, nonspecific binding of cells and free ATP to the beads may contribute both to the background and to variability in the signal. In fact, very high CV's were observed when the washing and assay solution was PBS which these were reduced when RPMI-1640 containing 10% FCS was used as a buffer. Additional improvements will include adding Hepes to the assay buffer to stabilize the pH in the absence of CO<sub>2</sub> and evaluating other additives such as mouse serum to further reduce the nonspecific binding and variability.

*b. Indirect vs. direct immunomagnetic separation.*

Most of the experiments were performed using an indirect procedure for reacting cells with beads. Cells were first incubated with an anti-CD4 antibody followed by incubation with goat anti-mouse IgG beads. This procedure was used because it gave more flexibility in the design of the experiments and the ability to examine different primary antibodies. Nonetheless, the assay sensitivity could probably be improved through the use of paramagnetic beads directly coupled with monoclonal antibody to CD4. When unstimulated mononuclear cells were separated using direct procedure (CD4 antibody directly coupled to beads), the signal to background ratio was almost 10 fold higher than that obtained in the two step assay (cells incubated with mouse monoclonal to CD4 first, then with beads coated with goat anti-mouse IgG). This difference was not accounted for by suboptimal levels of antibody or beads as increasing the concentration of either did not significantly affect the total signal or signal to noise ratio. The differences could be due to use of a superior antibody coupled to the beads or increased efficiency associated with direct binding. Development of beads with the appropriate cell surface chemistry will be an important component of the Phase II research and development.

*c. Different antibodies*

Several different antibodies were used in studies of activation. These included CD4, CD3, and CD69. CD4 antibody was chosen because of its ability to bind specifically to helper/inducer cells, cells that are likely to be stimulated first in a response to infectious agent or vaccine and that can provide input to both the humoral and cellular side of the immune response. CD3 antibody will bind to all T lymphocytes and is a general marker for cellular immune function. Finally, CD69 is an early stage activation antigen that appears on the cell surface rapidly after stimulation.

#### 4. Alternative methods for preparation of cells.

One of the goals in developing an assay to complement the standard lymphocyte proliferation assay was to reduce the steps required in performing the assay. In most lymphoproliferation formats, cells are separated from blood by density gradient centrifugation over Ficoll-Hypaque followed by a cell count and an adjustment in the cell number. Several alternative methods of preparing the cells were examined. For example, whole blood was diluted at different ratios in RPMI-1640 media, cultured in the presence of mitogens, separated by indirect immunomagnetic separation and measured by the ATP assay.

Several experiments were performed on whole blood in which the sample was diluted in PBS or media at different ratios, cells were isolated by immunomagnetic separation, then ATP was measured. An optimal ratio appeared to be about 1:10 of blood sample in media.

A major problem with this approach was that red blood cells and platelets were trapped in the bead pellet during these experiments. Because these elements contain significant ATP levels, contamination of the pellet with these components significantly affected the results. This was particularly a problem following culture of whole blood samples overnight and stimulation of samples with PHA. Improved cell separation techniques, in particular, a magnetic separator with an improved design may allow this approach to be retested. In addition, alternative culture media and solutions for washing beads may allow whole blood samples to be used.

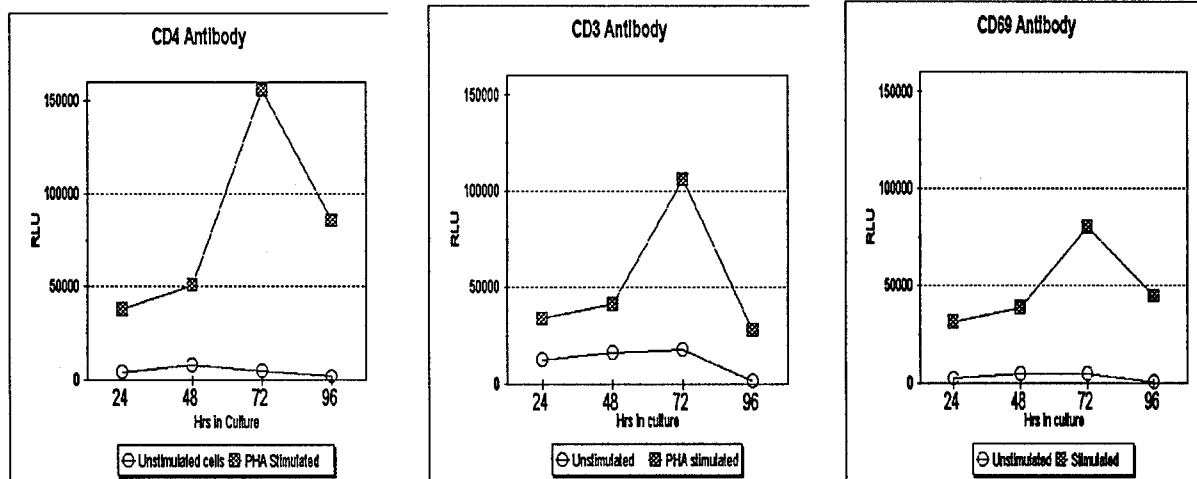
An alternative approach is to prepare total mononuclear cells by selective lysis of red blood cells followed by centrifugation for recovery of mononuclear cells. An ACK solution (solution of ammonium chloride (0.15 M), potassium carbonate (1.0 mM), and EDTA (0.1mM), pH 7.4) was prepared and used to treat whole blood samples. Removal of red blood cells was not complete, but lymphocyte viability was high and the immunomagnetic separation was improved. This approach needs to be more fully examined.

Another issue that remains to be fully resolved is how to account for the varying numbers of cells in different samples. The samples can be adjusted to a given cell concentration and this does increase the reproducibility but it is time consuming and may lead to inaccuracies.

#### 5. Assay performance.

Typical results for an experiment to test the time course of response based on separation using different primary antibodies are shown in Figure 3 below. In this experiment, cells were either unstimulated or stimulated with PHA for various time periods, then separated using one of three mouse monoclonal antibodies, CD4, CD3, or CD69. CD3 is the T3 antigen and consists of five different polypeptide chains with MWs ranging from 16-28 kD. These are physically associated with each other and with the T cell antigen receptor. Incubation of T cells with CD3 antibodies induces lymphoproliferation through similar, although not identical, paths as does antigen. CD3 antibodies binds to most T lymphocytes, CD4 expression is restricted to a subclass of lymphocytes that have helper cell function, and CD69 is a cell surface marker that appears early in lymphocyte activation. As has been seen in many other experiments, the response to PHA increases, then falls. While the maximum response for all three antibodies occurs at 72 hours, significant differences in the signal for stimulated and control cells are seen as early as 24 hours following stimulation. In this experiment, stimulation indexes of 9-15 were seen on day 1 increasing to over 50 on day 4 due in part to declines in the signals associated with the unstimulated cells.

Figure 3: Results from ATP assay using Immunomagnetic Separation  
Based on Different Cell Surface Markers



Cells were isolated from source leukocytes using density gradient centrifugation over Ficoll-Hypaque gradients and placed in RPMI-1640 containing 10 % FCS. Unstimulated cells were left in media alone. Stimulated cells were cultured in 1% PHA.

The results from these assays are expressed as relative light units and are based on photon counts from the luminometer for a period of time, in this case 10 sec. The counts can be measured in most luminometers over a wide dynamic range ( $10^0$ - $10^8$  light units) and the counts for a standard with a given concentration of LL are fairly constant. The total light obtained from a sample is dependent on the number of cells in the sample, the efficiency of capturing all the cells of a certain type from the sample, the concentration of LL and its activity, and the metabolic activity of the cells. The concentration of LL can be controlled and the LL activity can be made fairly stable in appropriate solutions for up to 5 days. To compensate for these differences, results could be expressed as stimulation indexes, which are values for stimulated relative to control cells after compensating for the background. Increase in the concentration of LL should have an impact of the sensitivity of the assay by increasing the signal without much impact on the background levels.

The background or noise of the assay is a critical parameter in assessing the sensitivity or signal:noise ratios. There are two types of background signal. That associated with the LL alone and that associated with incomplete separation of the cells. A negative control based on beads with different antibody has been used. Based on this type of background, stimulation indices for mitogens of 10-50 for mitogens and 2-10 for specific antigen have been observed after 24 hours of culture.

#### B. Use of the assay to measure CMI in Q Fever (Specific Aim #2).

A mouse model was used to assess the ability of the ATP assay to measure cellular immune function following vaccination. Balb/c mice were immunized with a total of 100 ug of a 1:1 mixture of Phase I and Phase II Coxiella burnetii antigen prepared from Nine mile strain (Integrated Diagnostics, Inc.) in complete Freund's adjuvant. Prebleeds were taken from the mice and did not react with

Q fever antigen even at 100 ug/ml. Bleeds taken at 7 days were tested for reactivity to Q fever antigen. Because of the small volume (approximately 100-200 ul) of whole blood that was obtained bleeds from pairs of mice were combined. Peripheral blood mononuclear cells were prepared by Ficoll-Hypaque gradients and cultured in RPMI-1640 containing 10% FCS in the presence or absence of a 1:1 mixture of Phase I and Phase II *C. burnetti* antigen. After 24 hours and 48 hours, ATP assays were performed on the cells. The table below clearly shows that the a response to *C. burnetti* antigen can be detected in PBMC from the immunized mice.

TABLE 1. **ATP ASSAY ON PBMC FROM MICE IMMUNIZED WITH Q FEVER ANTIGEN.**

Treatment	Day 1		Day 2	
	Total RLU	Index	Total RLU	Index
Control	8447	-	694	-
Q Antigen (I&II) - 10 ug/ml	22346	2.6	3206	4.6
Q Antigen (I&II) - 1 ug/ml	16566	1.8	1006	1.5
PHA - 1%	27442	3.3	9792	14.1

It should be noted that the overall signals are reduced on day 2. It is likely that this is due to decay in the activity of the luciferase upon storage at 4°C.

In a separate experiment, the response to separate Phase I and phase II antigens was determined. Cells were incubated with 10 ug/ml of either Phase I or Phase II antigen, no antigen, or with 1% PHA overnight then tested in the assay. The controls in the assay were not within appropriate levels as the cell concentration was low ( $< 1 \times 10^5$  cells/ml) and the signal from the PHA stimulated cells was not significantly greater than background. Nonetheless, the signal:background ratio for Phase I antigen was 2.00 whereas signal for Phase II antigen (1 ug/ml) under similar conditions was 1.12. These experiments need to be repeated with additional samples when the assay is optimized and also with multiple different samples from animals and humans at different stages after immunization or infection. Nonetheless, this type of differential response is consistent with what is known about the cellular response to *C. burnetti* antigens.

### **C. Comparison of the prototype assay with standard techniques for CMI (Specific Aim #3).**

The ATP assay was compared with other assays that have been used for measuring cellular immune function including assessment of lymphoproliferation to mitogens or antigens based on MTT, lymphoproliferation based on an ELISA to measure incorporation of BuDR into cellular DNA, and flow cytometry. Both the MTT assay and the BuDR assay required significantly higher cell concentrations to obtain a signal. In particular, in the MTT assay (based on a kit sold by Boehringer Mannheim) cells at concentrations of  $> 2 \times 10^6$ /ml or a total cell count of  $2 \times 10^5$  were just barely distinguishable from background. In addition, the total signal as measured spectrophotometrically for

even  $1 \times 10^7$  cells/ml was less than 0.600 O.D while the background for wells lacking cells was 0.200 O.D. so that the assay had little dynamic range. The BuDR ELISA assay was highly variable and no differences were detected between mitogen stimulated and control cells. This is probably due to a problem with the assay as measurement of DNA synthesis by  $^3\text{H}$ -thymidine uptake usually results in significant stimulation indexes for mitogens when  $1 \times 10^6$  cells/ml are cultured with mitogen for 3-5 days then pulsed for 4-12 hours with thymidine. The response to specific antigen is usually significantly lower (S.I. of 1-7 with maximum stimulation in 5-10 days).

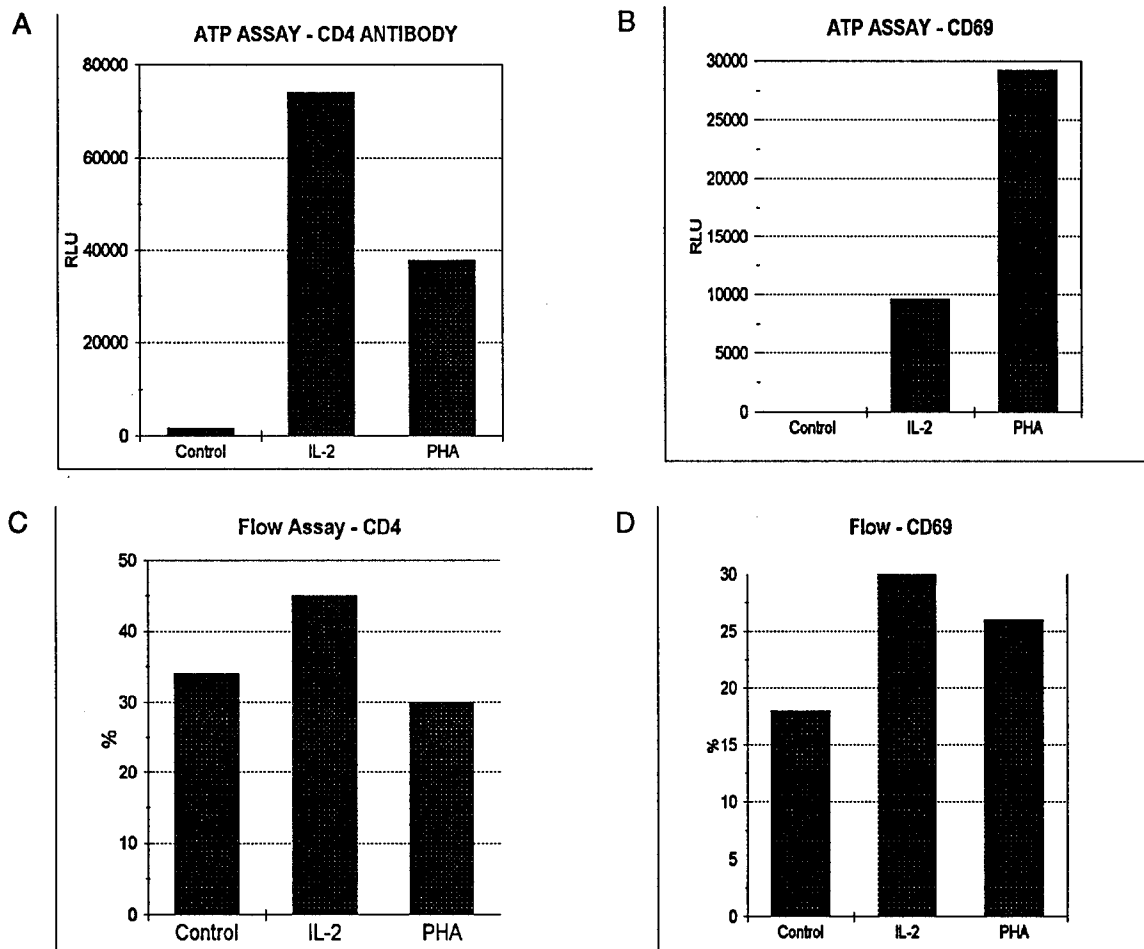
Mononuclear cells, either unstimulated or stimulated, were assessed by flow cytometry using CD4 antibody or CD69 antibody to measure numbers of activated cells. Figure 4 (next page) shows results of a comparison of flow data and the ATP assay on mononuclear cells that were unstimulated or stimulated with PHA, II-2. In this case cells were stimulated for 72 hours prior to assay. The analysis of the raw data from flow cytometry is much more complex than is the measurement of data from the luminescent assays. In particular, flow cytometry measures primarily the percentage of a particular part of a population that is stained with the antibody and is based on selection of certain populations based on size and density for measurement. The changes in the population measured by flow are of less magnitude than those measured by the ATP assay.

Additional experiments comparing the ATP based assay to traditional lymphoproliferative assays based on tritiated thymidine incorporation need to be completed particularly with Q fever antigen as the stimulus and with samples from mice or humans immunized with Q fever antigens to determine the relative performance of the different assays. Nonetheless, as was demonstrated above, responses to specific antigen and to mitogens can be detected in a reproducible manner within 24 hours of stimulation using the ATP assay and in lymphocytes from vaccinated mice only 7 days after a first immunization. The assay is clearly superior to the MTT assay and is more simple and rapid than is flow cytometry with significantly less complexity in the analysis of the data.

#### **D. Use of the assay with human samples (Specific Aim #4).**

Studies were not performed on human samples from individuals exposed to Q fever or vaccinated with Q fever antigens primarily because of the difficulty of obtaining these types of samples. Despite our own attempts and those of our technical grant officer, we were unable to obtain samples within the time period of the contract. There are few cases in the United States of Q fever, people are not routinely screened for exposure to Q fever as evidenced by persistence to antibody, and the vaccine trials that are ongoing are small involving only a few individuals. While there are periodic outbreaks of the disease in Europe and other sites outside the United States, measurement of cellular immunity requires a fresh blood sample drawn in an anti-coagulant maintained in a condition in which the cells remain viable. Because of the scarcity of the samples, it seemed prudent to wait to obtain samples until after the assay was fully optimized. Some sources for samples have now been identified; samples will be obtained from paid volunteers in vaccine trials for Q fever (Contact: Colonel Russell Byrne, Fort Detrick, Maryland), and individuals with evidence of prior exposure to Q fever are being identified in conjunction with trials for an antibody screening test for *C. burnetti* (Dr. Helen Paxton, Integrated Diagnostics, Inc.). Finally, researchers performing Q fever research from around the country are being canvassed for potential volunteers for screening.

Figure 4: Comparison of Results from ATP Assay and Flow Cytometry



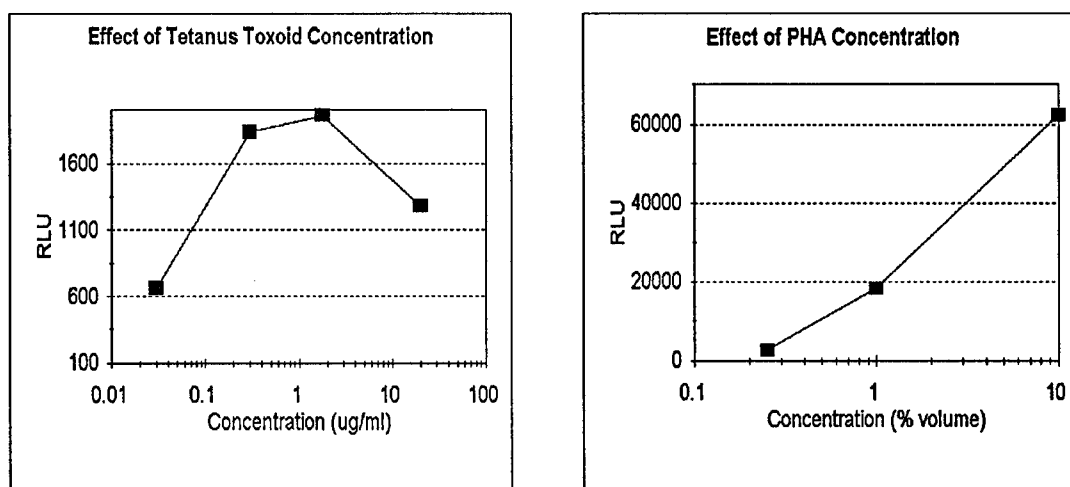
A&B are results from ATP assay of control (unstimulated), IL-2 (100 U/ml), or PHA (1%) after 24 hours of culture. Background was subtracted. B&C are flow cytometry results from the replicate cultures to those of A&B. Note that the % cells in flow cytometry are based on gating for lymphocytes on forward and side scatter bit maps and are not whole populations.

As an indication of the potential usefulness of the test for use in the detection of CMI responses in humans situations, the response to tetanus toxoid was measured. Most individuals have been vaccinated with tetanus toxoid and show lymphoproliferative responses to this antigen, although immunosuppressed individuals and a portion of the normal population do not respond. Previous studies have shown good correlation between *in vivo* delayed type hypersensitivity reactions and lymphoproliferative responses to tetanus toxoid (Borleffs et al, 1993), although the levels of response may vary considerably between individuals.

Normal PBMC from heparinized whole blood from paid volunteer donors or from source leu-

kocytes were isolated by density gradient centrifugation and resuspended at  $5 \times 10^5/\text{ml}$  in RPMI 1640 containing 10% FCS. Cells were stimulated either with varying levels of PHA or with tetanus toxoid (CalBiochem). The signal in the ATP assay was dependent on the dose of both mitogen, such as PHA, or antigen, such as tetanus toxoid as shown in Figure 4 below. These types of responses are expected for a lymphocyte response. The curve in the upper levels of tetanus toxoid may be related to a dose dependent toxicity or related to inhibition of cell activation by components in the toxoid formulation. This type of curve is frequently seen for mitogens or antigens in lymphoproliferation experiments.

Figure 5: Effect of Antigen or Mitogen Concentration on Signal in ATP Assay



PBMC isolated from source leukocytes were cultured in the presence of different levels of antigen(tetanus toxoid) or PHA for 24 hours then separated with CD4 antibody and magnetic beads and read with bioluminescence. The signal is expressed in relative light units.

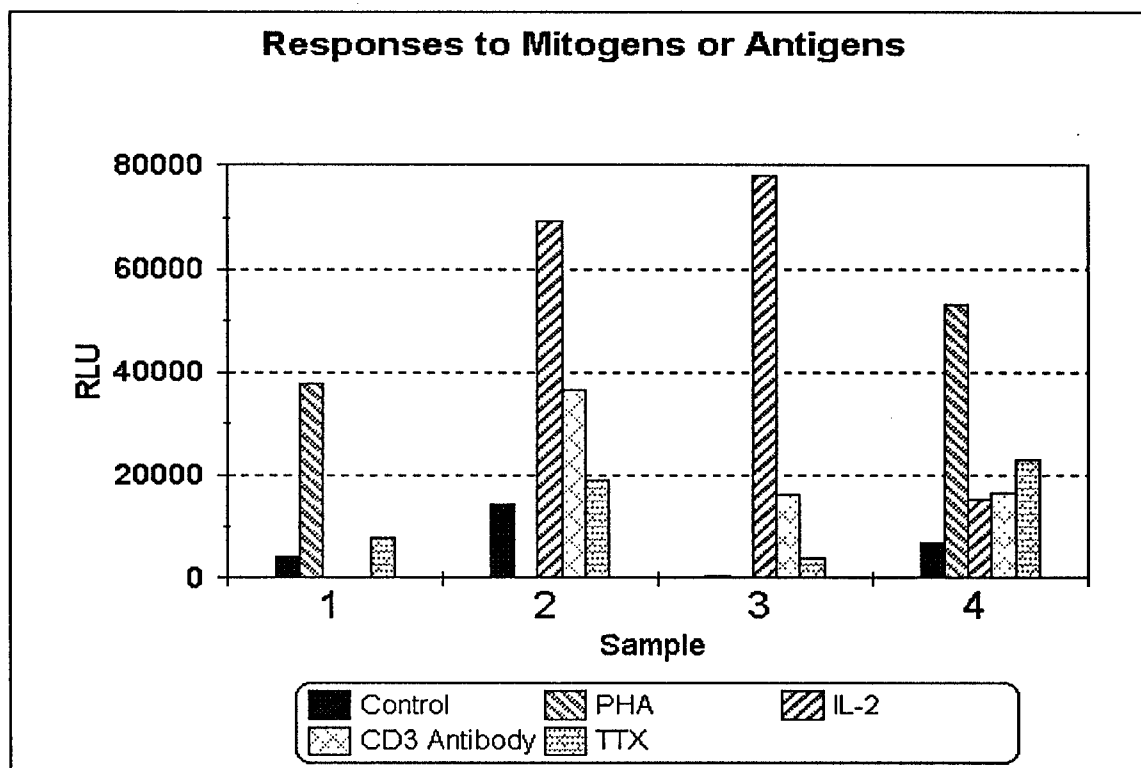
In the course of assay development, ATP assays were performed on PBMC isolated from several different individuals. These assays cannot be strictly compared because in each case the set of mitogens tested was different, and the conditions under which the tests were performed were slightly different. In some cases the stimulation conditions, such as the cell density, the time after isolation were also different. Figure 5 shows the raw data, essentially the relative light units for each sample and treatment condition. The graph shows that the assay is capable of detecting differences in unstimulated, control cells and mitogen stimulated cells even early after stimulation. In addition, the signals for antigen stimulated cells are smaller than those for mitogen stimulated cells but are consistently higher than those for the unstimulated cells (except for sample 3). A direct comparisons between the samples shown in the figure is not possible because of differences in the conditions of the assay when the samples were tested. Nonetheless, the responses to certain mitogens and to tetanus toxoid in these samples are very similar. The data does show that the assay is capable of



detecting responses to specific antigens and to mitogens in a short period of time in a sensitive and specific manner. Optimization of all the components of the assay should allow for more reproducible results to be obtained not only within a given experiment but also over time.

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Figure 6: Signals from Human Samples Cultured with Various Mitogens, Separated Immunomagnetically by CD4 Antibody and Measured in ATP Assay.



PBMC from normal human samples were incubated with different mitogens for 24 hours, separated by immunomagnetic separation using CD4 antibody, and tested by ATP assay. Results are expressed as relative light units. Samples were run at different times and came from different individuals.

## VIII. CONCLUSIONS

A prototype assay for measuring an aspect of cell mediated immunity has been developed. This assay is rapid, simple to perform, and quantitative. The assay requires little hands-on time and is more sensitive for measuring certain aspects of the response to mitogens or antigens than are other assays such as the XTT assay or flow cytometry. Lymphoproliferative assays may be more sensitive in certain situations and given larger stimulation indices but they are much more complex to perform, require at least three days of incubation, and use radioactive isotopes. In addition, the results in some cases have been highly variable between labs. The ATP assay can be developed in a kit format with all components included except for samples. This is likely to increase the reproduc-

ibility of results between labs and means the assay has the potential to be developed for use in a clinical lab setting.

The basic format for the prototype assay involves separation of peripheral blood mononuclear cells, culture of the cells either unstimulated or stimulated with antigen or mitogen for 24 hours, immunomagnetic separation of CD4<sup>+</sup> cells or other cell populations, cell lysis and measurement of ATP by bioluminescence utilizing the luciferin:luciferase reaction. Many of the critical parameters of the assay have been evaluated and defined. Some of the sources of assay variability have been defined and methods to reduce the variability identified. For example, the buffer used during the immunomagnetic step is critical not only for the binding but also for reduction in nonspecific binding.

The luciferin:luciferase reaction (LL) is a very sensitive measure of ATP concentration. In initial experiments, as few as 200 cells could be reliably distinguished from background following lysis of the cells, addition of a luciferin:luciferase mixture, and measurement in a luminometer. In most of the experiments performed in developing the prototype assay format, the LL mixture was diluted 1:10 to conserve reagent. Signals were sufficient even with diluted reagents to give good signals in most cases. However, because the signal increases with increasing LL concentration much faster than the background, increased LL concentration may allow detection of differences in smaller number of lymphocytes than were seen using the current method.

The ATP results may not be identical to measurements of lymphoproliferation in all cases because the signal is related to cell mass and cell metabolism both of which increase as increased numbers of cells proliferate, rather than to actual DNA synthesis. In some cases, induction of the early events of activation have not strictly correlated with completion of the process through the phase of DNA synthesis. In addition, recent research has indicated that activation of T cells is often associated with entry into an apoptosis. Thus, overall the biomass and metabolic rate could remain constant despite an increase in the proliferating cell population if a balanced number of cells are dying. The immunomagnetic separation should in part compensate for this if the appropriate antibodies are used.

The prototype has been used to demonstrate cellular immune responses in mice immunized with Q fever antigen. A cellular response was identified in the first bleed following immunization (at 7 days) and in subsequent bleeds. The response to phase II antigen was more prominent even though the mice were immunized with equal concentrations of both phase I and phase II antigens. Human samples have also been tested using the ATP assay for CMI to a recall antigen, tetanus toxoid, and responses were demonstrated in samples from several different individuals.

## **A. Improvements and future work**

### **1. Improvements in the basic format**

The prototype assay has been developed and its potential usefulness has been demonstrated in this phase of the work. Many aspects of the method need to be refined to increase the sensitivity and improve assay reproducibility.

#### ***a. Immunomagnetic separation***

The magnetic separation system used in the Phase I studies was not optimal for performing the assay because it was configured as a tube rack with the magnetic separator at the bottom of the tube. Because cells will settle under gravity at different rates depending on the cell density and the media composition, it is ideal to apply the magnetic field from a direction perpendicular to gravity,

that is from the side. Magnetic separators commercially available with laterally directed magnetic fields are designed to fit tubes with much larger volumes (15-50 ml) and accomodate only a few (3-5) tubes. In addition, these separators are designed so that the magnetic field is from one side only. An improved apparatus for separating the cells and for removing media must be designed to achieve optimum performance in a multi-assay setting.

#### *b. Cell preparation*

Use of a whole blood sample would be ideal from the perspective of simplicity and reduced hands-on time for an assay. Flow cytometry can be performed on whole blood samples and lymphoproliferation assays have also been performed using whole blood as a sample, although the results were not identical to those obtained using separated cells. Because red blood cells and platelets contain significant amounts of ATP, even a small amount of contamination of the cells separated using immunomagnetic beads with red blood cells or platelets can result in variable and spurious results. Several assays were performed on whole blood cells, but the results were highly variable and signals were quite high suggesting contamination of the final sample by platelets and red blood cells. In some cases, visible contamination of the final pellet with red blood cells could be observed. Improvements in the magnet separation apparatus and in the aspiration step may allow for use of a whole blood sample, but there may still be difficulties in culture, sample processing, and detection.

Most of the studies done in Phase I were done on cells isolated from blood by density gradient centrifugation. Mononuclear cell purification via density gradient separation has been the gold standard method for initially preparing cells for measuring various parameters of cellular immunity (Boyum, 1968). This method is tedious and time-consuming, may result in the loss of specific subsets of lymphocytes, and may lead to inconsistencies in the results due to incomplete recovery of cells from the blood. As an alternative, methods for preparing lysed whole blood cells have more recently been developed (Bray and Landay, 1989; Landay and Muirhead, 1989) for use primarily in flow cytometric studies of lymphocyte populations. These methods are simpler, require less hands on time, and less sample volume.

A number of commercial solutions are available for lysing red blood cells and for obtaining mononuclear preparations from whole blood. They contain formaldehyde which would interfere with the current assay, but a modification of these solutions could be examined as a simpler cell preparation method if it was not possible to use whole blood.

#### *c. Reagents*

Assay components can have a major effect on the sensitivity, specificity, and reproducibility of an assay. Each of the major components used in the assay needs to be evaluated in detail. For example, paramagnetic beads can be obtained in a variety of sizes ranging from 0.5-10 microns and are made of a variety of materials. Different types of beads have different densities and surface modifications that may affect the way they behave in the assay. The method of coupling antibody to the beads, the density of the coupled antibody, and the affinity of the antibody may be important in obtaining optimal results. Other critical reagents include the LL solution, the washing media, the culture media, and the lysing reagent.

## 2. Studies comparing this assay with other assays of CMI

A great deal of further comparison of the results of these assays with other measures of CMI is required to determine the performance and utility of this assay. These studies will need to be performed in a variety of model and real systems including human and animal diseases. The assay should be compared with lymphoproliferation, flow cytometry, and delayed-type-hypersensitivity to validate the use of this assay in research and clinical settings.

Other more basic studies need to be done to determine the nature of the response. It has been stated that the ATP levels can increase several logs in cells that have been activated from a resting state, yet this number is difficult to assess in cell populations. In a population of isolated cells, not all of the cells are resting at any given time and not all are activated simultaneously. Even in cloned populations not all of the cells are simultaneously activated. To address the nature of the ATP response on a basic level, it might be possible to evaluate lymphocyte populations transfected with the luciferase gene or loaded with luciferase protein and exposed to a mitogen or antigen along with luciferin. Using digital video imaging, it would be possible to obtain data from single cells in the population and to quantitate the levels of ATP as a function of changes in activation state. While these results would be useful to understanding the assay results on a mechanistic scale, these types of experiments are difficult to perform and often are complicated to interpret.

## 3. Extensions and expansions of the method

We performed some initial studies with immunomagnetic separation of cells using antibodies directed against other cell surface markers and showed that this was a feasible approach. The use of activation markers such as CD69, and early marker, or CD28 may give improved results in measuring responses to specific antigens. It may also be possible to evaluate minor subsets such as memory cells (CD45) and NK cells when the method is optimized.

Bioluminescence has been used to measure cytotoxicity as an alternative to chromium release in T lymphocytes by looking at the ATP in the media. It may be possible to modify the current assay to develop a method for measuring cytotoxicity.

Quantitation of the patterns of cytokines produced by lymphocytes under different conditions has been useful in evaluating the nature of the immune response and identification of Th1 and TH2 helper subtypes. An assay for quantification of intracellular cytokines using magnetic separation coupled with bioluminescent immunoassay technique could be developed that might be highly sensitive, easier to perform than flow cytometric assays of intracellular cytokines and much less expensive.

## 4. Applications of the methodology

This technology promises to be a powerful tool for measuring immune status parameters in a wide variety of conditions. Because CMI responses occur earlier than humoral responses in infectious disease, this technology can also be applied to diagnosis of infectious disease especially for organisms such as Q fever and tuberculosis where antibody responses may be difficult to detect or are nonspecific or the organism is difficult to isolate. The technology may also be useful in studying the response to vaccines and in evaluating the components of the cellular response over time following vaccination.

The assay may also be useful in clinical management and research in a number of other diseases. For example, this technology could potentially be used to monitor decline in immune function in AIDS patients long before current markers indicate any decline in cell numbers. Other diseases where immune status monitoring is critical to the appropriate management of care include transplantation, autoimmune disease, and chemotherapy for neoplastic disease. As a research tool, this technology will be useful in monitoring vaccine efficacy, in evaluation of the effectiveness of new immunotherapies including cancer vaccines and treatments for infectious disease, in immunotoxicology, in determining nutritional requirements for effective immune responses, and in research on the immune response to neoplasms and infectious diseases.

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**X. Raw Data - Copies of the raw data are available upon request.**

Received 2/8/00



DEPARTMENT OF THE ARMY  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

21 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information  
Center, ATTN: DTIC-OCA, 8725 John J. Kingman  
Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the attached Awards. Request the limited distribution statements for Accession Document Numbers listed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

FOR THE COMMANDER:

Encl  
as

A handwritten signature in cursive script, reading "Phylis Rinehart", is written over the typed name and title.  
PHYLIS M. RINEHART  
Deputy Chief of Staff for  
Information Management